FULL PAPER *Public Health*

Physiological Characterization of *Campylobacter jejuni* under Cold Stresses Conditions: Its Potential for Public Threat

Warangkhana CHAISOWWONG[1)], Akiko KUSUMOTO[1)], Miho HASHIMOTO[1)], Toshihiko HARADA[1)], Khuanwalai MAKLON[1)] and Keiko KAWAMOTO[1)]*

[1)]*Section of Food Microbiology and Immunology, Research Center for Animal Hygiene and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, 2–11 Inada, Obihiro, Hokkaido 080–8555, Japan*

(Received 27 June 2011/Accepted 19 August 2011/Published online in J-STAGE 2 September 2011)

ABSTRACT. *Campylobacter jejuni* is the major cause of human gastroenteritis worldwide. Under stress conditions, *C. jejuni* can enter a viable but non-culturable (VBNC) state. We found that the *C. jejuni* was able to enter a VBNC state by prolonged incubation at 4°C. The standard isolation methods using pre-enrichment steps in Bolton broth or Preston broth could not detect the VBNC cells in spiked chicken meat. The transcription levels of virulence-associated genes (*flaA, flaB, cadF, ciaB, cdtA, cdtB* and *cdtC*) were expressed in VBNC cells but in low levels. The VBNC cells retained the ability to invade Caco-2 human intestinal epithelial cells *in vitro*. In most cases, VBNC cells failed to resuscitate in Caco-2 cells, but in some experiments, they formed colonies after co-incubation with host cells. Collectively, *C. jejuni* enters into a VBNC state at 4°C and the VBNC *C. jejuni* remains virulent which may possibly lead to disease in humans. *C. jejuni* in VBNC state is a potential concern for food safety.

KEY WORDS: *Campylobacter jejuni*, resuscitation, viable but nonculturable state, virulence.

doi: 10.1292/jvms.11-0305; *J. Vet. Med. Sci.* 74(1): 43–50, 2012

Campylobacter jejuni, is a Gram-negative, microaerophilic, spiral shaped bacterium. It is the major cause of human gastroenteritis worldwide especially in developed countries [1, 15, 36, 37]. *Campylobacter* produce mild to severe diarrheal disease, and also can cause fever, nausea, and abdominal pain [49]. Although the illness is often self-limiting, it can lead to more severe consequences such as Guillain-Barré syndrome, acute demyelinating polyneuropathy, and reactive arthritis [22, 35]. Human campylobacteriosis is closely associated with the consumption of contaminated chicken meat, unpasteurized milk or untreated drinking water [23, 31, 48]. *C. jejuni* is mesophilic bacterium, grows best at temperatures ranging from 37°C to 42°C, and there are the reports that the survival of *C. jejuni* was lower under refrigeration temperatures [13, 18, 32].

In many countries, the cold chain is applied for over half the food consumed prior to reaching the consumer. In general, this storage method has been considered an effective means of reducing the possibility of infection, however, increasing demand for long-life fresh products creates new risk such as viable but non-culturable (VBNC) bacteria in the cold chain. A number of pathogens (e.g., *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Salmonella* Oranienburg, *Vibrio cholerae*, *Helicobacter pylori*, *Listeria monocytogenes*) enter a VBNC state in response to various environmental stresses such as cold exposure, pH, and high osmolarity [2, 3, 8, 34, 40]. In a VBNC state, the bacteria cannot be detected by conventional culture-based methods due to the loss of culturability on the media that normally support their growth, but remain viable and express various degrees of metabolic activities [12, 16, 37]. The VBNC could be set as the lost of culturability but the membrane integrity retained. In this state, culture-based enumeration of bacterial survival has significant limitations. Therefore, the bacteria at VBNC state are considered as a potential public health threat.

The VBNC phenomenon of *C. jejuni* was first described by Rollins and Colwell [45]. They found non-culturable *Campylobacter* cells after starvation (i.e. microcosm water) at 37°C. *C. jejuni* has been described to enter into the VBNC state in adverse environments such as pH stress, and combination of starvation and low temperature [4, 11, 14, 25, 32, 45]. However, it was uncertain whether VBNC *C. jejuni* retains virulency and become culturable in human cells.

The aims of this study were to examine whether VBNC *C. jejuni* could be detected by standard *Campylobacter* spp. isolation method. We also investigated the virulence of VBNC *C. jejuni* by analyzing the transcription levels of the virulence-associated genes and tested the ability of the VBNC cells to invade the human epithelial cells *in vitro*.

MATERIALS AND METHODS

Bacterial strain and culture preparation: *Campylobacter jejuni* CG8486 (serotype HS:4 CPS complex), a clinical isolate from a patient with inflammatory diarrhea in Thailand, was used in this experiment [42]. A loop of *C. jejuni* from a –80°C stock was streaked on modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (Oxoid, Hampshire,

* CORRESPONDENCE TO: KAWAMOTO, K., Section of Food Microbiology and Immunology, Research Center for Animal Hygiene and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, 2–11 Inada, Obihiro, Hokkaido 080–8555, Japan.
e-mail: kkeiko@obihiro.ac.jp

UK) at 42°C for 48 hr under microaerobic conditions by using a gas generating system (AnaeroPack-MicroAero, Mitsubishi Gas Chemical, Tokyo, Japan) that produced 5% O_2, 10% CO_2 and 85% N_2 in an anaerobic jar (Mitsubishi Gas Chemical). Well-isolated colonies were chosen and inoculated into 20 m*l* of Bolton broth (Oxoid) in 50 m*l* screw-cap tubes and incubated at 42°C under microaerobic conditions for 48 hr.

Induction of VBNC in C. jejuni: According to the previous study by Hazeleger [21] describing that *Campylobacetr* spp. entered VBNC state faster in nutrient-rich condition than in nutrient-deficient one when incubated at 4°C, we induced VBNC in *C. jejuni* by cold temperature incubation with nutrient-rich conditions in this study. To avoid the contamination of other bacteria during the long time incubation, we used *Campylobacter* selective nutrient media. One m*l* of bacterial culture was transferred into 15 m*l* tubes containing 12 m*l* of Bolton broth. A set of tubes were incubated at 4°C under microaerobic conditions. The enumeration of culturable, viable, and total cells was performed daily. For enumerating the culturable cells, the bacterial culture was centrifuged and the pellet was resuspended in 1 m*l* of sterile water. Then, a 0.1 m*l* aliquot and its 10-fold serial dilution series were spread on duplicate mCCDA plates and incubated at 42°C for 48 hr under microaerobic conditions. Colonies were then counted to calculate CFU/m*l*. Bacterial viability was determined by using the LIVE/DEAD *Bac*Light assay (Invitrogen, Carlsbad, CA). The percentage of viable cells was calculated from an evaluation of 1,000 cells. Total cells were determined by using a disposable plastic haemocytometer counting chamber (one-CellCounter, Erma Optical Works, Tokyo, Japan). In this study, we defined VBNC state as bacterial cells form no colonies on mCCDA yet the viability assessed by Baclight staining were greater than 60%. The VBNC cells incubated at 4°C for more than 38 days were used.

Detection VBNC Campylobacter in spiked chicken meat by standard isolation method: A piece of chicken was purchased from local supermarkets and aseptically divided into 25 g portions. For spiked experiments, the chicken meat was inoculated with stationary phase *C. jejuni* or VBNC *C. jejuni* which was induced by cold stress for more than 38 days. The chicken meat without bacterial inoculation was served as a control. Each 25 g of chicken meat was transferred into a stomacher filter bag, and 225 m*l* of Bolton broth, or 225 m*l* of Preston broth (Oxoid nutrient broth No. 2, supplement SR0117, and *Campylobacter* growth supplement SR 0232, with 5% lysed horse blood) was added. The chicken meat was homogenized for 1 min using a stomacher (AES CHEMUNEX, Cranbury, NJ). The mixture was incubated under microaerobic condition at 42°C for 48 hr. A 0.1 m*l* of the mixture and its 10-fold serial dilution were spread on mCCDA, then incubated at 42°C, microaerobic conditions for 48 hr before CFU were enumerated.

Real-time RT-PCR: Total RNA was extracted from the bacterial cells by a RiboPure-Bacteria kit (Ambion, Austin, TX) according to the manufacturer's instruction. The real time RT-PCR was performed using the One Step SYBR PrimeScript RT-PCR Kit (Takara, Shiga, Japan). The reactions were performed using a LightCycler 480 real-time PCR system (Roche Diagnostics, Rotkreuz, Switzerland). The primers were designed using DNASIS Pro Sequence analysis software (Hitachi Software Engineering, Tokyo, Japan) (Table 1). The expression level of virulence-associated genes (*flaA*, *flaB*, *ciaB*, *cadF*, *cdtA*, *cdtB* and *cdtC*) was calculated relatively against a calibration sample (*C. jejuni* at stationary phase), and an internal control gene (*rpoA*) was used to normalize the sample input amount, since *rpoA* gene has constant transcript levels under various stress conditions [43].

Immunostaining for invasive bacteria: The human colonic epithelial cell line Caco-2 was obtained from Riken Cell Bank (Tsukuba, Japan). Cells seeded onto a 12 mm-diameter round coverslips in 24 well culture plates at 1.0×10^5 cells/well were incubated with *C. jejuni* for 5 hr at 37°C to allow maximal bacterial invasion [46]. VBNC *C. jejuni* cells were prepared as described above by incubation at 4°C for more than 38 days. After washing the coverslips with

Table 1. Oligonucleotide primer sequences used for real-time RT-PCR analysis

Genes	Primers	n-mer	Sequence (5'-3')	PCR product (bp)
flaA	Cj-flaA_F2	22	GGATGGCGATAGCAGATAGTTT	113
	Cj-flaA_R2	22	CTCATCCATAGCCTTATCAGCA	
flaB	Cj-flaB_F1	20	ACACCAACATCGGTGCATTA	128
	Cj-flaB_R1	20	CATCCCTGAAGCATCATCTG	
cadF	Cj-cadF_F1	22	TTCTATGGTTTAGCAGGTGGAG	94
	Cj-cadF_R1	20	TTACACCCGCGCCATAAT	
ciaB	Cj-ciaB_F1	20	AAAAGCTTGGCAAGAAGCTG	107
	Cj-ciaB_R1	20	ATGCCACCGCATGAGTATAA	
cdtA	Cj-cdtA_F1	21	GGATTTGGCGATGCTAGAGTT	147
	Cj-cdtA_R1	20	CATTTGTGCGTGATTGCTTG	
cdtB	Cj-cdtB_F1	20	CTGGATGATAGCAGGGGATT	110
	Cj-cdtB_R1	20	CTTGAGTTGCGCTAGTTGGA	
cdtC	Cj-cdtC_F1	20	TCAGCTGTGCAAATTCGTTC	121
	Cj-cdtC_R1	22	AAATAGGATCTAGGGTGCAAGG	
rpoA	Cj_rpoA_F1	22	CTGTGGCTAAAATCAGTGCTTG	104
	Cj_rpoA_R1	22	TGGAGCATATCCTATGGTGCTA	

PBS, and the cells were fixed with 3.7% paraformaldehyde for 15 min. The extracellular bacteria were labeled by incubating with biotin-conjugated rabbit anti-*C. jejuni* polyclonal antibody (Abcam, Cambridge, UK) for 1 hr. The coverslips were washed and incubated with streptavidin conjugated Alexa Fluor 555 (Invitrogen). The cells were then permeabilized by 0.1% Triton X-100 in PBS, and were reincubated with biotin-conjugated rabbit polyclonal anti-*C. jejuni* to stain both intracellular and extracellular bacteria followed by streptavidin conjugated Alexa Fluor 488. By this differential staining, the extracellular bacteria showed reddish orange color as a result of double staining when the fluorescence images were merged, while intracellular bacteria showed green fluorescence. The coverslips were rinsed with water and mounted onto microscopic glass slides with Prolong Gold antifade reagent containing DAPI nuclei stain (Invitrogen).

Assay for intracellular culturable bacteria: The number of intracellular bacteria was determined by a gentamicin killing assay [19]. Caco-2 cells were infected with *C. jejuni* as described above. Non-adherent bacteria were removed by washing the cells with PBS three times. The extracellular bacteria were killed by the use of gentamicin (100 μg/m*l*) for 2 hr at 37°C. The bactericidal concentration required for complete killing was determined before the experiments. After washing, the medium was replaced with MEM with 10% FBS. The Caco-2 cells were lysed by 0.1% Triton X-100 in PBS at 24 hr and 48 hr post inoculation. Enumeration of intracellular culturable bacteria was performed on mCCDA agar.

RESULTS

VBNC induction of C. jejuni at 4°C: The culturability, viability, and total cell count of *C. jejuni* at 4°C were routinely determined to assess whether the bacteria entered into VBNC state. As shown in Fig. 1, the number of CFU was decreased day by day. No colonies were detected at day 38, whereas the total cell counts were maintained substantially constant throughout the incubation period. The viability of *C. jejuni* was 79.14% at day 38, indicating that *C. jejuni* entered the VBNC state after prolonged incubation at 4°C. In addition, morphological appearance was observed that the of VBNC cells from spiral form to coccoid form as described in previous studies (data not shown) [21].

VBNC C. jejuni detection in food by standard isolation method: We examined whether the VBNC *Campylobacter* cells in food could be detected by a standard isolation method in spiked experiments. The chicken meat was contaminated with VBNC cells or stationary cells, and then standard isolation methods using two different enrichment procedures were performed. The VBNC *C. jejuni* was induced by exposure to cold stress for 45 days (the viability was 72%). Since the VBNC cells do no form colonies, to adjust the size of inoculum between stationary and VBNC cells, viable cells were calculated from the number of total cells and the percentage of viable cells as described in Materials and Methods. Same number of viable cells were inoculated in food, then determined the number of CFU by plating on mCCDA after enrichment process. No *Campylobacter* was detected from unspiked chicken meat (data not shown). Increased number of bacteria was detected from stationary *C. jejuni*-spiked meat by both Bolton and Preston enrichment methods (Table 2). On the other hand, no *Campylobacter* cells were recovered from meat that inoculated with VBNC cells. These results indicated that neither the use of Bolton nor Preston enrichment step could resuscitate VBNC cells to culturable ones.

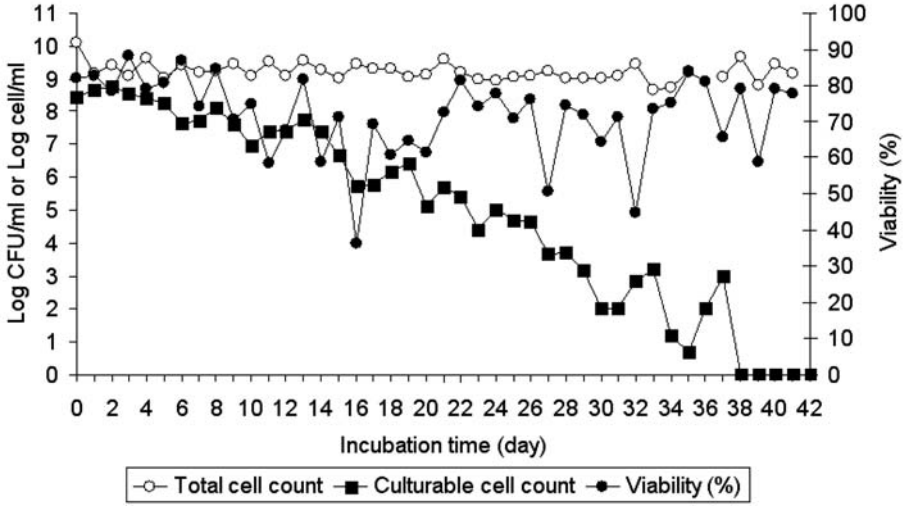

Fig. 1. *Campylobacter jejuni* CG8486 strain enters into a VBNC state after incubation at 4°C. Total cell counts (Log_{10}cells/m*l*) (open circle), culturable cell counts (Log_{10}CFU/m*l*) (closed square) and the viability percentages (closed circle) are shown. These data are a representative replicates from three independent experiments.

Table 2. Number of detectable *C. jejuni* by quantitative *Campylobacter* isolation method using Bolton and Preston enrichment media

Media	Inoculation bacteria	No. of bacteria	
		Inoculum[a)] per g	Detectable bacteria (CFU/g)
Bolton broth	Stationary	5.0×10^3	3.2×10^6
	VBNC	5.0×10^3	0.0
Preston broth	Stationary	5.0×10^3	9.8×10^5
	VBNC	5.0×10^3	0.0

a) Viable cells (calculation by total cell count and viability percentage).

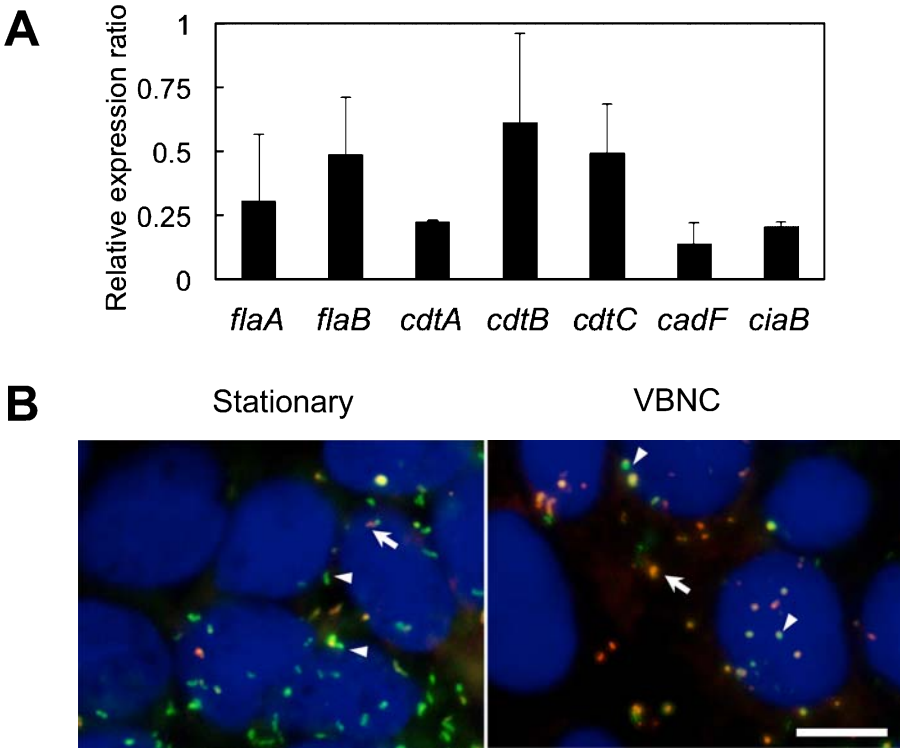

Fig. 2. Relative expression levels of virulence associated genes in VBNC *C. jejuni* compared to those of stationary *C. jejuni* cells (A). Invasive *Campylobacter* cells in human intestinal epithelial cells (B). Caco-2 cells were infected with stationary *C. jejuni* cells (left panel), or VBNC cells (right panel) for 2 hr. The arrowheads indicate invasive bacteria whereas the arrow indicates extracellular bacteria. N: Caco-2 nuclei were fluoresced blue from DAPI staining. The bar indicates 20 μm.

Transcriptions of virulence-associated genes in VBNC C. jejuni: To check whether the VBNC cells retain the virulence, we investigated the transcription of virulence-associated genes (*flaA*, *flaB*, *cdtA*, *cdtB*, *cdtC*, *cadF* and *ciaB*) by RT real-time PCR. These virulence genes were expressed in VBNC state of *C. jejuni* cells exposed cold stress for 38 days (Fig 2A). The relative expression ratio comparing with stationary phase in *flaA*, *flaB*, *cdtA*, *cdtB*, *cdtC*, *cadF* and *ciaB* were 0.31, 0.49, 0.22, 0.61, 0.49, 0.14, and 0.21 times, respectively, indicating that expression of all tested virulence genes were down-regulated, but maintained in VBNC cells.

Invasion of VBNC C. jejuni in human epithelial cells: Above results suggest that the virulence of *C. jejuni* may be attenuated in VBNC state. Although *C. jejuni* is considered to be primarily an extracellular pathogen, its abilities to adhere, invade in intestinal epithelial cells is also believed to be part of its pathogenesis. A number of studies *in vivo* have established that *C. jejuni* is a facultatively intracellular bacterium [17, 28, 46, 50]. The virulence genes tested above were considered to be important for adhesion and invasion to host intestinal tract. Thus, we examine invasion ability of

VBNC bacteria in host cells. Caco-2 cells were infected with either stationary *C. jejuni* or VBNC ones for 2 hr, since [46] showed that maximal invasion of *C. jejuni* was maintained between 1 and 2.5 hr post inoculation in this cell line. The invasive bacteria were visualized by the immunofluorescence assay as described in Materials and Methods. As shown in Fig. 2B, the intracellular spiral form bacteria were observed in host cells that infected with stationary *C. jejuni* (left panel). The intracellular bacteria were also detected in VBNC-infected Caco-2 cells (Fig. 2B, right panel). The morphology of VBNC *C. jejuni* in host cells was coccoid shape and different from that of stationary bacteria.

These results raised a question that intracellular VBNC *C. jejuni* was resuscitated to become culturable in host cells. To examine this possibility, the gentamycin killing assay was performed to determine the number of culturable bacterial cells in the Caco-2 cells. Bacterial colonies were detected from stationary inoculation as described in many studies. For example, when Caco-2 cells were inoculated with 3.0×10^7 CFU/well stationary *C. jejuni*, the number of intracellular bacteria was 1.2×10^5 CFU/well at 24 hr and increased at 48 hr such as 2.1×10^7 CFU/well (data not shown). We repeated the assay 10 times, no colonies were detected at 24 hr in VBNC *C. jejuni.* In most cases, culturable bacteria were not observed during 72 hr incubation in VBNC cell inoculation. However, in two experiments, colonies were detected on agar plates from Caco-2 lysate that inoculated with VBNC bacteria (data not shown). VBNC bacteria was inoculated into Caco-2 culture with initial inoculum of 1.1×10^7, or 1.5×10^8 viable cells/well. After 48 hr post inoculation, 5.0 CFU/well or 105 CFU/well were detected from infected Caco-2 cells, respectively. The recovered number of bacteria was quite lower than stationary bacteria.

DISCUSSION

In this study, we examined the effect of refrigeration temperature on *C. jejuni* culturability under nutrient-rich and microaerobic conditions. After 38 days incubation, the *Campylobacter* cells were difficult to recover on agar plates, yet approximately 70% of the cells stained as viable by BacLight analysis. These results show that exposure to low temperature induced the VBNC state in *C. jejuni* in spite of sufficient nutrient availability. This suggests that cold stress by itself can induce the VBNC state in *C. jejuni*. Virulence gene expression in VBNC *C. jejuni* has not been definitively clarified. To elucidate the potential virulence of VBNC cells, we demonstrated the transcription of virulence genes including *cadF*, *ciaB*, *flaA*, *flaB*, *cdtA*, *cdtB* and *cdtC*. These virulence genes are significantly involved in disease pathogenesis. The outer membrane protein, CadF, mediates the binding of *C. jejuni* to fibronectin of host cells [39]. CiaB, the Campylobacter invasion antigen B, is similar to type III secretion proteins and it is required for invasion [30]. The study by Wassenaar *et al.* [52] showed that flagella play a role in bacterial internalization most likely due to the need for flagellin (FlaA/FlaB). The cytolethal distending toxin (CdtA, CdtB and CdtC) causes eukaryotic cells to arrest in the G_2/M phase of the cell cycle, leading to cell death [53]. The expression levels of all tested virulence genes at VBNC state were decreased when compared with stationary phase. The cold temperature stress might induce the decrease of virulence gene expressions. Moen *et al.* [38] investigated global gene expression patterns of *C. jejuni* that was cultured at 5°C up to 7 days, and they found the expression of flagellin genes, *flaA* and *flaB*, were down-regulated. In contrast, a large number of genes involved in energy metabolism increased at 5°C than 25°C, indicating that *C. jejuni* may require a greater energy to survive under cold temperature. Therefore, bacteria might down-regulate and control transcription of virulence genes that are unnecessary for survival under stressful environment.

The ability of pathogenic *C. jejuni* to adhere and invade into intestinal mucosa is a prerequisite for infection. The decrease in transcription of virulence genes led to a question about bacterial interaction ability to host cells. As shown in Fig 2B, intracellular VBNC cells was detected in Caco-2 cells. However, in most of gentamycin killing assays, no detectable colonies were appeared in Caco-2 cell lysate that were inoculated VBNC *C. jejuni*. These results indicate that VBNC *C. jejuni* may restore the ability to adhere to host cells but failed to resuscitate in hostile envionment. But in few results, VBNC bacteria became culturable after co-cultured with Caco-2 cells. The culture medium without Caco-2 cells did not support the resuscitation of VBNC cells (data not shown), suggesting that the resuscitation occurred by the interaction with host cells, not by a factor of supplement medium. Since the number of culturable bacteria in host cells was significantly lower in VBNC *C. jejuni* compared to stationary cells, the virulence of bacteria might be severely attenuated in VBNC state. This idea was agreed with the facts that all tested virulence genes were down-regulated in VBNC cells (Fig. 2A). According to previous studies by Konkel *et al.* [29, 30], *C. jejuni* synthesizes and secretes proteins upon co-culture with mammalian cells to stimulate the bacterial entry into host cells, and no bacteria were detected within host epithelial cells that exposed to the heat-killed organisms which is metabolically inactive. It seems that *C. jejuni* that is metabolically active and secrete proteins from the flagellar for maximal invasion of host epithelial cells. To clarify the invasive property of VBNC cells, it requires further studies.

Mechanisms of "resuscitation" (i.e. induction of re-culturable state) of *Campylobacter* cells are not understood. It has been reported that the *in vivo* resuscitation occurred by inoculation of non-culturable *C. jejuni* to 1-day chicks or embryonated eggs [4, 11]. Jones *et al.* [25] demonstrated that a suckling mouse passage of non-culturable cells induced intestinal bacterial colonization. Our results and the previous studies imply that co-presence of eukaryotic cells stimulates a resuscitation process of VBNC *C. jejuni*. There is a possibility that resuscitation might be induced by host-derived signals. Several host-derived molecules are

sensed by bacteria. For example, eukaryotic hormone epinephrine/norephinephrine stimulate growth and virulence gene expression in pathogenic bacteria [5, 7]. At this moment, we can't rule out the possibility that VBNC *C. jejuni* may restore the ability to shift from non-culturable state to culturable one in host cells. The further study for identification and purification of host factors is required in order to understand the resuscitation mechanism, but also to give a clue to develop methods for detecting VBNC bacteria.

The detection of a bacterial cell has been traditionally determined by its ability to grow and make colonies on agar plates. The outbreaks and sporadic cases of campylobacteriosis have frequently been reported that *Campylobacter* was isolated from patients. But it is often the case that the bacteria could not be isolated from suspected food or environmental samples [23, 26, 33, 47]. There are many reasons for failure to identify the source of contamination, however it could be partly explained by the probable contamination of VBNC bacteria. In this study, we examined whether or not the VBNC *C. jejuni* was detected by the standard isolation procedures. In general, the optimal isolation of *Campylobacter* requires combination of enrichment process and special selective agars. Various selective enrichment media have been developed such as Bolton, Preston, Exeter, Park and Sanders, and modified Brucella broth [6, 10, 27, 51]. Among them, Bolton and Preston broths are more commonly used worldwide for the detection of *Campylobacter* spp. [20, 24]. Bolton broth is known to be useful for the recovery of injured cells [6, 41], while Preston broth is the most selective and highest isolation rate medium [6, 9]. However, the standard isolation methods using these media could not resuscitate and detect VBNC cells. Although our results do not rule out the possibility that other enrichment media can detect the VBNC *C. jejuni*, our study indicate that standard methods for the detection of *Campylobacter* may give false negative results if VBNC cells are present in samples. The cause of disease associated with VBNC *C. jejuni* might be underestimated. These findings tell us that culture-based methods for detection and enumeration of food-borne bacteria may have limitations. A number of PCR assays for detecting small number of *Campylobacter* spp. have been developed, however, most of them can work in combination with an enrichment culture step. Since VBNC cells could not be resuscitated and multiply in common enrichment media, a new technique to recover them should be developed in future.

In conclusion, *C. jejuni* enters into a VBNC state by cold stress under nutrient conditions, and the VBNC *C. jejuni* remains potentially virulent and can be resuscitated by host cells. During the past decade, *Campylobacter* spp. became a major cause of human enteritis worldwide. A small number of *C. jejuni* are necessary to cause illness, according to the human feeding study [44]. Because VBNC *C. jejuni* cells can resuscitate and remain pathogenic under favorable conditions, which make them potential threats to the human health, it is of major importance to develop a new procedure for detection and isolation of them from food, drinking water and environmental sources.

ACKNOWLEDGMENTS. This work was supported by a grant from Ministry of Education, Culture, Science and Technology (MEXT), by a Grant-in-Aid for Scientific Research (21651074) from the Japan Society for the Promotion of Science (JSPS) and by a grant from the Ministry of Health, Labour, Welfare, Japan.

REFERENCES

1. Adak, G.K., Meakins, S.M., Yip, H., Lopman, B.A. and O'Brien, S.J. 2005. Disease risks from foods, England and Wales, 1996–2000. *Emerg. Infect. Dis.* **11**: 365–372.
2. Adam, B.L., Bates, T.C. and Oliver, J.D. 2003. Survival of *Helicobacter pylori* in a natural fresh water environment. *Appl. Environ. Microbiol.* **69**: 7462–7466.
3. Asakura, H., Ishiwa, A., Arakawa, E., Makino, S.-I., Okada, Y., Yamamoto, S. and Igimi, S. 2006. Gene expression profile of *Vibrio cholerae* in the cold stress-induced viable but non-culturable state. *Environ. Microbiol.* **9**: 869–879.
4. Baffone, W., Casaroli, A., Citterio, B., Pierfelici, L., Campana, R., Vittoria, E., Guaglianone, E. and Donelli, G. 2006. *Campylobacter jejuni* loss of culturability in aqueous microcosms and ability to resuscitate in a mouse model. *Int. J. Food Microbiol.* **107**: 83–91.
5. Bansal, T., Englert, D., Lee, J., Hegde, M., Wood, T.K. and Jayaraman, A. 2007. Differential effects of epinephrine, norepinephrine, and indole on *Escherichia coli* O157:H7 chemotaxis, colonization, and gene expression. *Infect. Immun.* **75**: 4597–4607.
6. Baylis, C.L., MacPhee, S., Martin, K.W., Humphrey, T.J. and Betts, R.P., 2000. Comparison of three enrichment media for the isolation of *Campylobacter* spp. from foods. *J. Appl. Microbiol.* **89**: 884–891.
7. Belay, T. and Sonnenfeld, G. 2002. Differential effects of catecholamines on *in vitro* growth of pathogenic bacteria. *Life Sci.* **71**: 447–456.
8. Besnard, V., Federighi, M., Declerq, E., Jugiau, F. and Cappelier, J.M. 2002. Environmental and physico-chemical factors induce VBNC state in *Listeria monocytogenes*. *Vet. Res.* **33**: 359–370.
9. Bolton, F.J., Coates, D., Hinchliffe, P.M. and Robertson, L. 1983. Comparison of selective media for isolation of *Campylobacter jejuni/coli*. *J. Clin. Pathol.* **36**: 78–83.
10. Bolton, F.J. and Robertson, L. 1982. A selective medium for isolating *Campylobacter jejuni/coli*. *J. Clin. Pathol.* **35**: 462–467.
11. Cappelier, J.M., Magras, C., Jouve, J.L. and Federighi, M. 1999. Recovery of viable but non-culturable *Campylobacter jejuni* cells in two animal models. *Food Microbiol.* **16**: 375–383.
12. Chaiyanan, S., Huq, A., Maugel, T. and Colwell, R.R. 2001. Viability of the nonculturable *Vibrio cholerae* O1 and O139. *Syst. Appl. Microbiol.* **24**: 331–341.
13. Chan, K.F., Tran, H.L., Kanenaka, R.Y. and Kathariou, S. 2001. Survival of clinical and poultry-derived isolates of *Campylobacter jejuni* at a low temperature (4°C). *Appl. Environ. Microbiol.* **67**: 4186–4191.
14. Chaveerach, P., ter Huurne, A.A.H.M., Lipman, L.J.A. and van Knapen, F. 2002. Survival and resuscitation of ten strains of

Campylobacter jejuni and *Campylobacter coli* under acid conditions. *Appl. Environ. Microbiol.* **69**: 711–714.
15. Coker, A.O., Isokpehi, R.D., Thomas, B.N., Amisu, K.O. and Obi, C.L. 2002. Human campylobacteriosis in developing countries. *Emerg. Infect. Dis.* **8**: 237–243.
16. Colwell, R.R., Brayton, P., Herrington, D., Tall, B., Huq, A. and Levine, M.M. 1996. Viable but non-culturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. *World J. Microbiol.* **12**: 28–31.
17. Day, W.A. Jr, Sajecki, J.L., Pitts, T.M. and Joens, L.A. 2000. Role of catalase in *Campylobacter jejuni* intracellular survival. *Infect. Immun.* **68**: 6337–6345.
18. El-Shibiny, A., Connerton, P. and Connerton, I. 2009. Survival at refrigeration and freezing temperatures of *Campylobacter coli* and *Campylobacter jejuni* on chicken skin applied as axenic and mixed inoculums. *Int. J. Food Microbiol.* **131**: 197–202.
19. Elsinghorst, E.A. 1994. Measurement of invasion by gentamycin resistance. *Methods Enzymol.* **236**: 405–420.
20. FDA BAM, 1998. FDA Bacteriological Analytical Manual. Chapter 7, *Campylobacter*. <http://www.cfsan.fda.gov/~ebam/bam-7.html>.
21. Hazeleger, W.C., Janse, J.D., Koenraad, P.M., Beumer, R.R., Rombouts, F.M. and Abee, T. 1995. Temperature-dependent membrane fatty acid and cell physiology changes in coccoid forms of Campylobacter jejuni. *Appl. Environ. Microbiol.* **61**: 2713–2719.
22. Hannu, T., Mattila, L., Rautelin, H., Pelkonen, P., Lahdenne, P., Siitonen, A. and Leirisalo-Repo, M. 2002. *Campylobacter*-triggered reactive arthritis: population-based study. *Rheumatology* **41**: 312–318.
23. Heuvelink, A., Heerwaarden, C.V., Zwartkruis-Nahuis, A., Tilburg, J.J.H.C., Bos, M.H., Heilmann, F.G.C., Hofhuis, A., Hoekstra, T. and de Boer, E. 2009. Two outbreaks of campylobacteriosis associated with the consumption of raw cows' milk. *Int. J. Food Microbiol.* **134**: 70–74.
24. ISO, 1995. Microbiological of food and animal feedings stuffs-Horizontal method for the detection of thermotolerant *Campylobacter*. ISO 10272: 1995 (E). International Standards Organization, Geneva.
25. Jones, D.M., Sutcliffe, E.M. and Curry, A. 1991. Recovery of viable but non-culturable *Campylobacter jejuni*. *J. Gen. Microbiol*. **137**: 2477–2482.
26. Jones, G. and Roworth, M. 1996. An outbreak of *Escherichia coli* O157 and campylobacteriosis associated with contamination of a drinking water supply. *Public Health* **110**: 277–282.
27. Josefsen, M.H., Lübeck, P.S., Aalbaek, B. and Hoorfar, J. 2003. Preston and Park-Sanders protocols adapted for semi-quantitative isolation of thermotolerant *Campylobacter* from chicken rinse. *Int. J. Food Microbiol.* **80**: 177–183.
28. Konkel, M.E., Hayes, S.F., Joens, L.A. and Cieplak, W. Jr. 1992. Characteristics of the internalization and intracellular survival of *Campylobacter jejuni* in human epithelial cell cultures. *Microb. Pathog.* **13**:357–370.
29. Konkel, M.E. and Cieplak, W. Jr. 1992. Altered synthetic response of *Campylobacter jejuni* to cocultivation with human epithelial cells is associated with enhanced internalization. *Infect. Immun.* **60**: 4945–4949.
30. Konkel, M.E., Klena, J.D., Rivera-Amill, V., Monteville, M.R., Biswas, D., Raphael, B. and Mickelson, J. 2004. Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. *J. Bacteriol.* **186**: 3296–3303.
31. Kuusi, M., Nuorti, J.P., Hänninen, M.-L., Koskela, M., Jussila, V., Kela, E., Miettinen, I. and Ruutu, P. 2005. A large outbreak of campylobacteriosis associated with a municipal water supply in Finland. *Epidemiol. Infect.* **133**: 593–601.
32. Lázaro, B., Cárcamo, J., Audícana, A., Perales, I. and Fernández-Astorga, A. 1999. Viability and DNA maintenance in non-culturable spiral *Campylobacter jejuni* cells after long-term exposure to low temperatures. *Appl. Environ. Microbiol.* **65**: 4677–4681.
33. Lehner, A., Schneck, C., Feierl, G., Pless, P., Deutz, A., Brandl, E. and Wagner, M. 2000. Epidemiologic application of pulsed-field gel electrophoresis to an outbreak of *Campylobacter jejuni* in an Austrian youth centre. *Epidemiol. Infect.* **125**: 13–16.
34. Liu, Y., Wang, C., Tyrrell, G., Hrudey, S.E. and Li, X.-F. 2009. Induction of *Escherichia coli* O157:H7 into the viable but non-culturable state by chloraminated water and river water, and subsequent resuscitation. *Environ. Microbiol.* **1**: 155–161.
35. McCarthy, N. and Giesecke, J. 2001. Incidence of Guillain-Barré syndrome following infection with *Campylobacter jejuni*. *Am. J. Epidemiol.* **153**: 610–614.
36. Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5**: 607–625.
37. Ministry of Health, Labour and Welfare, Japan, 2010. *Campylobacter* enteritis in Japan, 2006–2009. Infectious Agents Surveillance Report 31. <http://idsc.nih.go.jp/iasr/31/359/tpc359.html>.
38. Moen, B., Oust, A., Langsrud, Ø., Dorrell, N., Marsden, G.L., Hinds, J., Kohler, A., Wren, B.W. and Rudi, K. 2005. Exploration multifactor approach for investigating global survival mechanisms of *Campylobacter jejuni* under environmental conditions. *Appl. Environ. Microbiol.* **71**: 2086–2094.
39. Monteville, M.R., Yoon, J.E. and Konkel, M.E. 2003. Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer membrane protein and microfilament reorganization. *Microbiology* **149**: 153–165.
40. Panutdaporn, N., Kawamoto, K., Asakura, H. and Makino, S.-I. 2006. Resuscitation of the viable but non-culturable state of *Salmonella enterica* serovar Oranienburg by recombinant resuscitation-promoting factor derived from *Salmonella* Typhimurium strain LT2. *Int. J. Food Microbiol.* **106**: 241–247.
41. Paulsen, P., Kanzler, P., Hilbert, F., Mayrhofer, S., Baumgartner, S. and Smulders, F.J.M. 2005. Comparison of three methods for detecting *Campylobacter* spp. in chilled or frozen meat. *Int. J. Food Microbiol.* **103**: 229–233.
42. Poly, F., Read T., Tribble, D.R., Baqar, S., Lorenzo, M. and Guerry, P. 2007. Genome Sequence of a Clinical Isolate of *Campylobacter jejuni* from Thailand. *Infect. Immun.* **75**: 3425–3433.
43. Ritz, M., Garenaux, A., Berge, M. and Federighi, M. 2009. Determination of *rpoA* as the most suitable internal control to study stress response in *C. jejuni* by RT-qPCR and application to oxidative stress. *J. Microbiol. Methods* **76**: 196–200.
44. Robinson, D.A. 1981. Infective dose of *Campylobacter jejuni* in milk. *Br. Med. J* . **282**: 1548.
45. Rollins, D.M. and Colwell, R.R. 1986. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Appl. Environ. Microbiol.* **52**: 531–538.
46. Russel, R.G. and Blake, D.C. 1994. Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. *Infect. Immun.*

62: 3773–3779.
47. Sacks, J.J., Lieb, S., Baldy, L.M., Berta, S., Patton, C.M., White, P.M., Bigler, W.L. and Witte, J.J. 1986. Epidemic campylobacteriosis associated with a community water supply. *Am. J. Public Health* **76**: 424–429.
48. Saito, S., Yatsuyanagi, J., Harata, S., Ito, Y., Shinagawa, K., Suzuki, N., Amano, K.-I. and Enomoto, K. 2005. *Campylobacter jejuni* isolated from retail poultry meat, bovine feces and bile, and human diarrheal samples in Japan: comparison of serotypes and genotypes. *FEMS Immunol. Med. Microbiol.* **45**: 311–319.
49. Skirrow, M.B. 1977. Campylobacter enteritis: a "new" disease. *Br. Med . J.* **2**: 9–11.
50. Szymanski, C.M., King, M., Haardt, M. and Armstrong, G.D. 1995. *Campylobacter jejuni* motility and invasion of Caco-2 cells. *Infect. Immun.* **63**: 4295–4300.
51. Wang, W.-L.L., Luechtefeld, N.W., Reller, L.B. and Blaser, M.J. 1980. Enriched Brucella medium for storage and transport of cultures of *Campylobacter fetus* subsp. *jejuni*. *J. Clin. Microbiol.* **12**: 479–480.
52. Wassenaar, T.M., van der Zeijst, B.A., Ayling, R. and Newell, D.G. 1993. Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. *J. Gen. Microbiol.* **139**: 1171–1175.
53. Whitehouse, C.A., Balbo, P.B., Pesci, E.C., Cottle, D.L., Mirabito, P.M. and Pickett, C.L. 1998. *Campylobacter jejuni* cytolethal distending toxin causes a G_2-phase cell cycle block. *Infect. Immun.* **68**: 184–191.